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Evidence for a 90 kDa Heat-Shock Protein Gene Expression in the Amphibian Oocyte

PASCAL COUMAILLEAU, BERNARD BILLOUD, PATRICIA SOURROUILLE, NICOLE MOREAU, AND NICOLE ANGELIER[1]

Laboratoire de Biologie Moléculaire et Cellulaire du Développement, UA 1135 CNRS Groupe "Gènes et Développement," Université Pierre et Marie Curie, Bâtiment C, 6ème étage, 9 quai Saint Bernard, 75252 Paris cedex 05-France

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In order to study expression of a 90-kDa heat-shock protein during amphibian oogenesis at physiological temperature, we isolated a *Pleurodeles waltl* hsc90 cDNA by screening an ovarian cDNA library with a chicken hsp90 cDNA probe. The cDNA thus obtained—named Pw90—shows a high homology level with the hsp90 gene in other species. RNase protection analysis led us to conclude that this sequence is part of the cognate gene hsc90 and is constitutively expressed in oocytes. Furthermore, results of quantitative Northern blot analysis, as well as *in situ* hybridizations on oocyte sections or lampbrush chromosome spreads, provide evidence for expression of hsc90 transcripts at every stage of oogenesis. Moreover, they point to the fact that an accumulation of transcripts occurs very early in oogenesis. Simultaneously, the expression of HSC90-related protein was analyzed on Western blots using a monoclonal antibody (AC88) and a polyclonal antibody (AP90Ct) raised against the *Pleurodeles* C-terminal part of HSC90. We provide evidence for a net accumulation of HSC90-related protein in oocytes. Immunolocalization shows that a nuclear transfer occurs in the course of oogenesis and leads to a concentration equilibrium between cytoplasm and nucleus in stage VI oocytes.

INTRODUCTION

During the course of oogenesis, transcriptional and translational activities lead to the storage of maternal products necessary for early embryogenesis (for review, see Davidson, 1986). Such transcriptional and translational processes are known to be controlled by protein factors synthesized in the cytoplasm and subsequently transferred to the nucleus. Molecular chaperones such as heat shock proteins have been shown to interfere with the nuclear transport of these protein factors (for review, see Hendrick and Hartl, 1993). In somatic cells, heat shock proteins act to modify or maintain the conformation of proteins passing across intracellular membranes; in particular, they facilitate transport through the nuclear membrane. Heat shock proteins are thus assumed to interfere indirectly with events involved in the control of gene expression. In certain systems, such as that of oocytes, which present intense transcriptional activity, it can be hypothesized that heat shock proteins are likewise involved in control of maternal gene expression. Among these proteins, HSP90 is one of the most thoroughly studied and is the most abundant heat shock protein. Synthesis of HSP90 is increased in cells submitted to stress conditions, but also occurs in uninduced cells, i.e., under normal conditions (HSC90; Lai *et al.*, 1984). This protein is highly conserved between species and its expression has been reported in a wide variety of eucaryotic stressed and unstressed cells from yeast, flies, chickens, mice, and humans (Lindquist and Craig, 1988; Hickey *et al.*, 1989). It is an ubiquitous protein which is essential to the survival of cells, since deletion of the two genes encoding HSP82 and HSC82 in the yeast *Saccharomyces cerevisiae* is lethal (Borkovich *et al.*, 1989), but its exact functions are presently unknown. HSP90 interacts with several viral oncogene products that display tyrosine kinase activity (Brugge *et al.*, 1981; Lipsich *et al.*, 1982; Opperman *et al.*, 1981; Ziemiecki *et al.*, 1986). It also associates with tubulin and actin (Nishida *et al.*, 1986; Sanchez *et al.*, 1988) and stimulates the activity of eucaryotic initiation factor 2α subunit-specific protein kinase (Rose *et al.*, 1987). In mammalian cells, where it has been extensively studied, HSP90 has been proven to be a component of steroid receptor heterocomplexes (Pratt, 1987, 1990). In particular, evidence is emerging that suggests that members of the HSP90 class act as molecular chaperones in the mechanism of signal transduction by steroid receptors. In the absence of steroid hormones, the steroid receptor binds to HSP90 and is unable to activate transcription of steroid-controlled genes. Addition of steroid hormone displaces bound HSP90 and produces a receptor capable of activating transcription (Groyer *et al.*, 1987; Sanchez *et al.*, 1987; Willmann and Beato, 1986; Klein-Hitpass *et al.*,

[1] To whom correspondence should be addressed.

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1990). Moreover, genetic studies in yeast suggest that receptors can be activated by hormone only if they are initially bound to HSP90 (Picard *et al.*, 1990). HSP90 protein, therefore, interacts with target proteins involved in various cellular processes. In oocytes, HSC90-related proteins could also interact with target proteins directly involved in molecular processes controlling gene expression.

To address the questions of HSC90 function during development, we analyzed hsc90 expression in the amphibian oocyte under physiological conditions. The present study provides evidence for constitutive expression of hsc90 genes in the amphibian oocyte. A partial hsc90 cDNA sequence enabled us to analyze mRNA expression throughout oogenesis using Northern and *in situ* hybridization. Simultaneously, an antibody was raised against a synthetic peptide deduced from the previously recovered nucleotide sequence. Results from HSC90 expression studies by both Western blotting and immunofluorescence on oocyte sections show that HSC90 is expressed throughout oogenesis. In early oogenesis, this protein is preferentially cytoplasmic, while in late (stage VI) oocytes, its nuclear concentration clearly increases. These results are discussed in relation to the possible functions of HSC90 in oocytes.

MATERIALS AND METHODS

Animals and Tissues

Ovary and liver were isolated from *Pleurodeles waltl* (Amphibian, Urodele) raised in our laboratory at the physiological temperature of 20°C. Oocytes were defolliculated either manually under the microscope using fine forceps or by treatment with collagenase as previously described (Moreau and Boucher, 1981; Moreau *et al.*, 1991). These defolliculated oocytes were then transferred to modified Barth's medium (MBS, Gurdon, 1976) and sized according to the six stages described by Bonnanfant-Jaïs and Mentré (1983). The absence of follicular cells was checked by microscopic examination of Hoechst 33258 (Sigma)-stained oocytes.

Screening of an Ovarian cDNA Library and Sequence Analysis

A cDNA library was previously made from poly(A)$^+$ RNA prepared from ovary using the λgt11 cloning system with an oligo(dt) primer (Billoud *et al.*, 1993). The library was screened using the 2.4-kb *Bssh*II/*Mae*I fragment of a chicken hsp90 cDNA (Catelli *et al.*, 1985). Positive clones were subcloned into pGEM7Zf(+) and sequenced on both strands by the chain termination method (Sanger *et al.*, 1977). A positive clone called Pw90 contained a large fragment of the *Pleurodeles* hsp90 cDNA. Pw90/571, a 571-bp *Xho*I/*Cla*I restriction fragment, was used for subsequent molecular analysis.

Northern Blot, Quantitative RNA Slot Blot Hybridization, and RNase Protection Analysis

Total RNA was isolated from ovary or oocytes by the LiCl-urea method (Auffray and Rougeon, 1980). Poly(A)$^+$ and poly(A)$^-$ RNA were isolated by the chromatography affinity method described by Aviv and Leder (1972). In inducibility experiments, tissues or cells (ovary, liver, or oocytes) were placed in 80% culture Medium 199, 25 m*M* Hepes (Gibco) at normal temperature (20°C) or at 34°C for 2.5 hr; then, RNA was extracted as described above.

Northern blotting was performed according to Sambrook *et al.* (1988). RNA samples were electrophoresed in denaturing 0.8% agarose gel, transferred onto a nitrocellulose membrane (Schleicher & Schuell BA85), and baked for 2 hr under vacuum at 80°C. The filter was prehybridized in 0.2 ml/cm^2 of 50% formamide, 6× SSC, 2× Denhardt's, 1 μg/ml sonicated salmon sperm DNA for 4 hr at 65°C. Hybridization was performed in the same buffer plus a denatured double-stranded [^{32}P]DNA Pw90/571 probe whose specific activity was typically 0.5 to 2 × 10^9 cpm/μg (Amersham random priming kit). Washing was for 2× 30 min at 65°C in 1× SSC, 0.1% SDS and for 2× 30 min in 0.1× SSC, 0.1% SDS. 28S and 18S ribosomal RNAs were used as size standards to determine the approximate molecular weight of transcripts.

RNase protection analysis was performed as described by Krieg and Melton (1987) with minor modifications. cDNA fragments were cloned into pGEM7Zf (+) to direct the *in vitro* synthesis of antisense transcripts using T7 RNA polymerase (Boehringer-Mannheim, Germany) in the presence of [α-^{32}P]CTP (800 Ci/mmole, Amersham, UK). Full-length probe was purified from a 0.3 *M* sodium acetate, 2 m*M* EDTA, 0.5% SDS, 20 μg/ml tRNA polyacrylamide gel by elution at 37°C. Hybridizations were carried out in 40 m*M* Pipes, pH 6.4, 400 m*M* NaCl, 1 m*M* EDTA at 45°C for 24 hr. The samples were digested by 20 U/ml T1 RNase at 37°C for 1 hr. Following phenol chloroform extraction and ethanol precipitation, the protected fragments were resolved by electrophoresis on a 5% polyacrylamide gel and exposed to X-ray film.

RNA quantitation experiments were derived from the technique described by Taylor *et al.* (1986) and modified by Billoud *et al.* (1993). Total RNA was extracted from various oocyte stages by the method described above. In order to estimate extraction efficiency, we added to the grinding buffer a radioactively labeled RNA (an hsp90-unrelated [^{35}S]RNA) enabling deposit on the membrane of total RNA in an amount corresponding to a fixed num-

ber of oocytes whatever the extraction yield. The exact amount of hsp90 mRNA in oocytes was determined by comparing signals obtained by hybridization of the Pw90/571 probe to endogenous oocyte RNA and to a synthetic sense Pw90/571 RNA used as a standard for the estimation of the copy number (10^4 to 10^8 copies).

After hybridization with the [^{32}P]Pw90/571 probe and autoradiography, the signal was measured by optically scanning densitometry on a Biocom image processing device. In practice, both synthetic RNAs used in extraction and in standardization were transcribed *in vitro* with low specific activity [^{35}S]rUTP to prevent a background signal.

In Situ Hybridization on Oocyte Sections and Lampbrush Chromosomes

Ovary was processed for *in situ* hybridization using several fixation methods. Pieces of ovary were fixed in (a) freon which was then substituted by ethanol (−20°C), (b) 4% cold formaldehyde in PBS for 1 hr, or (c) cold methanol/acetone (−20°C). After several washes, the tissue was embedded in cytoparaffin and 10-μm-thick sections were collected on gelatin-coated glass slides.

The protocol used for *in situ* hybridization on ovary sections was basically that of Wilkinson (1987). A single-stranded RNA labeled with [^{35}S]rUTP was synthesized *in vitro* using Pw90/571 inserted in pGEM7zf(+) as a template and Sp6 or T7 RNA polymerase (Promega). The probe (3×10^8 cpm/μg) was hydrolyzed to an average size of 100 bases and dissolved in hybridization buffer (50% formamide; NaCl 0.3 *M*; Tris-HCl 20 m*M*, pH 7.4; EDTA 5 m*M*; phosphate buffer 10 m*M*, pH 8; 10% dextran sulfate; 1× Denhardt's; 0.5 mg/ml yeast tRNA; 10 m*M* DTT) to a final concentration of 12×10^5 cpm/μl (20 μl/slide). Hybridization was carried out overnight at 50°C. Washing was for 30 min at 50°C in 5× SSC, 10 m*M* DTT, for 20 min at 65°C in 50% formamide, 2× SSC, 10 m*M* DTT and for 2× 10 min at 37°C in washing solution (4 *M* NaCl, 100 m*M* Tris-HCl, pH 7.5, 50 m*M* EDTA). To reduce background, slides were treated with 20 μg/ml of RNase A and 1 U/ml of RNase T1 (diluted in washing buffer) for 30 min at 37°C. Slides were washed again for 5 min at 37°C in 2× SSC and for 5 min at 37°C in 0.1× SSC, dehydrated, and air dried. Slides were dipped into a Kodak NTB2 emulsion and kept at 4°C for 1 week until development. Ovary sections were stained with May-Grunwald/Giemsa.

In situ hybridization on *P. waltl* lampbrush chromosome spreads was carried out as previously described (Penrad-Mobayed *et al.*, 1991). Lampbrush chromosomes were prepared according to Angelier *et al.* (1986). Germinal vesicles of oocytes were manually isolated (Gall, 1954), the nuclear envelope was removed, and the nuclear content centrifuged (1500*g* for 30 min at 8°C) onto the coverslip. Chromosome preparations were fixed in 70% ethanol, dehydrated, washed in xylene to remove paraffin, and air dried of acetone. [^{35}S]UTP RNA probes were synthesized as described above and dissolved in hybridization buffer (40% formamide 4× SSC, 100 m*M* DTT, 100 m*M* $NaPO_4$, pH 7, 300 μg/ml yeast tRNA, 300 μg/ml *Escherichia coli* DNA) to a final concentration of 8×10^4 cpm/μl (5 μl/slide). Hybridization was carried out overnight at 42°C and washed for 3× 20 min at 60°C in 0.1× SSC, 10 m*M* DTT, dehydrated, and air dried. Lampbrush chromosome preparations were stained with 0.1% Coomassie blue solution (Gall *et al.*, 1981).

Antibodies

The monoclonal antibody AC88, prepared against a male-specific antigen derived from the water mold *Achlya ambisexualis*, was a gift of Dr. D. O. Toft (Mayo Clinic, Minnesota). It recognizes the cytosolic HSP90-free form and was essentially used in Western blotting immunodetections under denatured conditions (Riehl *et al.*, 1985).

A polyclonal antibody specific for *Pleurodeles* HSC90 was generated. It was called AP90Ct. A 15-aa peptide (TTDEIPPLEEDEDAS), named p90Ct, from the C-terminal region of *Pleurodeles* HSC90 sDNA was synthesized (Neosystem). This peptide sequence was determined using CITI2 software which predicts antigenic determinants (Fig. 1). Antiserum was raised in New Zealand rabbits (Dr J. C. Mazié, Hybridolab, Institut Pasteur, Paris). Prior to injection, rabbits were bled for preimmune serum.

Protein Extraction, Western Blotting, and Protein Analysis

Following defolliculation, batches of 20 defolliculated oocytes were homogenized in Tris-EDTA buffer (Chen and Stumm-Zollinger, 1986). The homogenate was centrifuged for 10 min at 10,000*g* and proteins of the supernatant were precipitated overnight at −20°C by 9 vol of ethanol. For compartimentalization analysis, germinal vesicles and cytoplasms were manually separated (Angelier *et al.*, 1986) and prepared for subsequent electrophoresis as previously described (Moreau *et al.*, 1991) except for centrifugation, which was 10,000*g* for 10 min. Total oocyte, nuclear, or cytoplasmic proteins were separated in 7.5% polyacrylamide gels, either in one (Laemmli, 1970) or two dimensions (O'Farrell, 1975) and then electrophoretically transferred to an Immobilon-PVDF membrane (Millipore). Blots were treated with antibodies (AC88 or AP90Ct) as previously described (Moreau *et al.*, 1986). Bound antibodies were detected by incubation

with a peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit antibody. In quantitative experiments, total extracts corresponding to a fixed number of oocytes, cytoplasms, or nuclei were run on and blotted as described above. Immunodetection was performed under unsaturated conditions using AC88 and a ^{125}I-labeled anti-mouse antibody (specific activity 3000 Ci/mmole, Amersham). The signal was quantified by both densitometry scanning and γ counting of corresponding cut-out filter pieces.

Immunocytochemistry

Immunocytochemistry experiments were carried out by indirect immunofluorescence: 7.5-μm-thick sections of ovary fixed with Romeis fixative and embedded in polyester wax (Hausen *et al.*, 1985) were prepared as previously described (Moreau *et al.*, 1986). Sections were overlaid with 1% BSA in PBS for 30 min and then incubated with the polyclonal antibody against *Pleurodeles* HSC90 (1:500 dilution in 1% BSA, PBS) or with the monoclonal antibody AC88 (100 μg/ml). After three washes in PBS, slides were incubated with a fluorescein isothiocyanate-conjugated goat anti-mouse (Miles Scientific, Paris) or anti-rabbit antibody (ICN ImmunoBiologicals) diluted 1:100 in PBS, 1% BSA, washed, and mounted in Mowiol.

RESULTS

Expression of an hsc90 mRNA in Ovaries of the Amphibian P. waltl

A chicken hsp90 cDNA (Catelli *et al.*, 1985) was used to screen a *Pleurodeles* ovarian cDNA library (Billoud *et al.*, 1993). One cDNA clone (Pw90) containing a 2.1-kb insert was recovered. The nucleotide sequence of Pw90 (Fig. 1) exhibited very strong homology with hsp90 sequences of other species, and analysis of the deduced amino acid sequence revealed that Pw90 encoded for a polypeptide closely related to the carboxy terminal part of the HSP90 protein family (Fig. 1). Comparison with the previously characterized HSP90 sequences in other species also provided evidence for very strong homology with mammalian HSP90, particularly in the mouse and in humans (92.1%) (Table 1).

In order to determine whether the Pw90 sequence was a partial sequence of a heat-inducible (hsp) or constitutive gene (hsc), RNase protection analysis was carried out in normal and heat-shocked somatic (liver) cells. An 891-base single-stranded antisense RNA probe was generated from a *Nae* digest from the plasmid containing Pw90/571 (571 bp). Figure 2A shows that hybridization with RNA from normal liver cells led to the protection of about 570 bases of the probe, i.e., almost the same size as Pw90/571. The same fragment is protected under stressed conditions but the intensity of the signal did not increase. These results led us to conclude that Pw90/571 was strictly constitutive in somatic cells and was not heat-inducible, which therefore strongly suggested that Pw90/571 was part of the *Pleurodeles* hsc90 gene. Furthermore, the same results were obtained in ovaries, i.e., in oocytes with follicular cells (Fig. 2A).

Using this partial cDNA sequence as a probe, we analyzed the expression of Pw90 mRNA under physiological conditions in *Pleurodeles* ovaries. Total, poly(A)$^+$ and poly(A)$^-$ RNA were extracted and then probed under high-stringency conditions with Pw90/571. Pw90/571 was found to hybridize to a single transcript of about 3 kb in total and poly(A)$^+$ RNA, but not in poly(A)$^-$ RNA (Fig. 2B). These results therefore provided evidence for the presence of a hsc90 mRNA in ovaries of the amphibian *P. waltl*.

Expression of hsc90 mRNA in Oocytes

RNase protection analysis shows that hybridization of the same probe as that used for somatic cells (891/ bases) to total RNA from defolliculated oocytes led to the protection of the same 571-base fragment as in somatic cells (Fig. 2A) and provided evidence for an hsc90 mRNA expression in oocytes.

In order to analyze hsc90 mRNA expression during oogenesis, Northern blots were carried out with total RNA from collagenase defolliculated oocytes at defined stages (from stage II to stage VI). The absence of follicular cells was rigorously checked by microscopic examination of Hoechst 33258 (Sigma)-stained oocyte. One unique hsc90 mRNA of about 3 kb was detected for each stage (Fig. 3A), i.e., the same size as that of the poly(A)$^+$ hsc90 RNA detected in total ovary (see above). Quantitation of hsc90 mRNA could therefore be efficiently undertaken by slot blot hybridization of total oocyte RNA using Pw90/571 as a probe according to the procedure described under Materials and Methods. Scanning densitometry analysis of slot blot hybridization of autoradiograms (Figs. 3B and 3C) provided evidence for a very

FIG. 1. Nucleotide sequence of *P. waltl* hsc90 cDNA clone (Pw90) along with its predicted amino acid sequence. Two restriction sites are underlined in the nucleotide sequence, successively *Xho*I and *Cla*I. The 15-amino-acid peptide p90Ct, used for synthesis of *Pleurodeles*-specific anti-HSC90 antibody is underlined in the carboxyl terminal region of the protein sequence. Nucleotide and amino acid numbers are shown on the right.

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AAA GAG GAC CAG AGC GAG TAC CTG GAG GAG AAG CGT GTC AAG GAG GTG GTG AAG AAG CAC TCC CAG TTT ATC GGT   75
 K   E   D   Q   S   E   Y   L   E   E   K   R   V   K   E   V   V   K   K   H   S   Q   F   I   G   25

TAC CCC ATC ACC CTT TAC CTG GAG AAG GAG CGC GAG AAG GAG ATC AGC GAC GAC GAG GCC GAG GAG GAA AAG GTG  150
 Y   P   I   T   L   Y   L   E   K   E   R   E   K   E   I   S   D   D   E   A   E   E   E   K   V   50

GAG GAG AAG AAA GAC GAA GAG GAG GCC CCC AAG GAG GAG GAG GAC AAG CCC AAG ATC GAG GAC GTG GGC TCG GAC  225
 E   E   K   K   D   E   E   E   A   P   K   E   E   E   D   K   P   K   I   E   D   V   G   S   D   75

GAC GAG GAG GAC GGC AAG GAG AAG AAG AAG AAG AAG AAG ACG AAG AAG ATC AAG GAG AAG TAC ATC GAC CAG GAG GAG  300
 D   E   E   D   G   K   E   K   K   K   K   K   T   K   K   I   K   E   K   Y   I   D   Q   E   E  100

CTG AAC AAG ACC AAG CCC ATC TGG ACG AGC AAC CCG GAC GAC ATC AGC CAG GAG GAG TAC GGG GAG TTC TAC AAG  375
 L   N   K   T   K   P   I   W   T   S   N   P   D   D   I   S   Q   E   E   Y   G   E   F   Y   K  125
                                                                  XhoI
AGC CTG ACC AAC GAC TGG GAG GAC CAC CTG GCC GTC AAG CAC TTC TCG GTG GAG GGC CAG CTC GAG TTC CGC GCT  450
 S   L   T   N   D   W   E   D   H   L   A   V   K   H   F   S   V   E   G   Q   L   E   F   R   A  150

CTC CTC TTC ATC CCC GGC CGA GCG CCC TTC GAC CTC TTC GAG AAC AAG AAG AAA AAG AAC AAC ATC AAG CTG TAC  525
 L   L   F   I   P   G   R   A   P   F   D   L   F   E   N   K   K   K   K   N   N   I   K   L   Y  175

GTG CGC CGA GTC TTC ATC ATG GAC AGC TGC GAC GAG CTC ATC CCG GAG TAC CTG AAC TTC GTG CCC GGC GTG GTG  600
 V   R   R   V   F   I   M   D   S   C   D   E   L   I   P   E   Y   L   N   F   V   P   G   V   V  200

GAC TCG GAG GAC TTG CCC CTG AAC ATC TCC CGC GAG ATG CTG CAG CAA AGC AAG ATC CTG AAG GTC ATC CGC AAG  675
 D   S   E   D   L   P   L   N   I   S   R   E   M   L   Q   Q   S   K   I   L   K   V   I   R   K  225

AAC ATC GTC AAG AAG TGT GAG CTC ACC TTC TGC GAG CTG GCC GAG GAC AAG GAC AAC TAC AAG AAG TTC TAC GAG  750
 N   I   V   K   K   C   E   L   T   F   C   E   L   A   E   D   K   D   N   Y   K   K   F   Y   E  250

GCC TTC TCC AAG AAC CTC AAG CTT GGA ATC CAC GAG GAC TCT GCC AAC CGG AAG AAG CTG TCA GAG CTG CTG CGT  825
 A   F   S   K   N   L   K   L   G   I   H   E   D   S   A   N   R   K   K   L   S   E   L   L   R  275

TAC CAC ACC TCC CAG ACT GGT GAT GAG ATG GCA TCT CTC ACA GAA TAC GTC TCC CGC ATG AAG GAG ACC CAG AAG  900
 Y   H   T   S   Q   T   G   D   E   M   A   S   L   T   E   Y   V   S   R   M   K   E   T   Q   K  300

GCC ATA TAT TAC ATC ACT GGA GAG AGC AAG GAG CAA GTG GCA AAC TCT GCG TTT GTG GAG CGT GTC CGG AAG CGC  975
 A   I   Y   Y   I   T   G   E   S   K   E   Q   V   A   N   S   A   F   V   E   R   V   R   K   R  325
                                 ClaI
GGC TTC GAG GTG GTG TAC ATG ACC GAA CCC ATC GAT GAG TAC TGT GTC CAG CAG TTG AAG GAG TTT GAT GGA AAG 1050
 G   F   E   V   V   Y   M   T   E   P   I   D   E   Y   C   V   Q   Q   L   K   E   F   D   G   K  350

ACC CTC GTC TCT GTG ACC AAG GAG GGC CTG GAG CTG CCA GAG GAT GAG GAA GAA AAA AAG AAA ATG GAG GAG AGC 1125
 T   L   V   S   V   T   K   E   G   L   E   L   P   E   D   E   E   E   K   K   K   M   E   E   S  375

AAA TCC AAG TTC GAG AAC CTG TGC AAG CTT ATG AAG GAG ATT TTG GAC AAG AAG GTT GAG AAG GTG ACT GTT TCC 1200
 K   S   K   F   E   N   L   C   K   L   M   K   E   I   L   D   K   K   V   E   K   V   T   V   S  400

AAC CGC CTT GTC TCT TCG CCC TGC TGC ATT GTG ACC AGT ACC TAT GGG TGG ACC GCC AAC ATG GAA AGG ATT ATG 1275
 N   R   L   V   S   S   P   C   C   I   V   T   S   T   Y   G   W   T   A   N   M   E   R   I   M  425

AAG GCA CAG GCT CTT CGC GAC AAC TCC ACC ATG GGC TAC ATG ATG GCC AAG AAG CAC TTA GAA ATC AAC CCT GAC 1350
 K   A   Q   A   L   R   D   N   S   T   M   G   Y   M   M   A   K   K   H   L   E   I   N   P   D  450

CAC CCA ATT GTG GAG ACC CTC CGG CAG AAG GCG GAG GCA GAT AAA AAT GAC AAA GCG GTT AAG GAC CTT GTA GTC 1425
 H   P   I   V   E   T   L   R   Q   K   A   E   A   D   K   N   D   K   A   V   K   D   L   V   V  475

CTA CTG TTT GAG ACG GCG CTG CTG TCA TCT GGC TTC TCT CTG GAA GAC CCC CAG ACG CAC TCA AAC CGC ATG TAC 1500
 L   L   F   E   T   A   L   L   S   S   G   F   S   L   E   D   P   Q   T   H   S   N   R   M   Y  500

AGA ATG ATC AAG CTT GGG TTA GGA ATT GAT GAG GAT GAA GTT GCT GTA GAG GAA CCT GTA GCT GCC ACG ACA GAT 1575
 R   M   I   K   L   G   L   G   I   D   E   D   E   V   A   V   E   E   P   V   A   A   T   T   D  525

GAG ATC CCA CCT CTG GAG GAG GAT GAA GAT GCA TCC CGA ATG GAG GAG GTT GAT                              1629
 E   I   P   P   L   E   E   D   E   D   A   S   R   M   E   E   V   D                              543
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p90Ct

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taaagcactgcaggctgtctaacaccaggcatgctgcggcaatttttcaaaatgactccttgcaattgctacacgcctctgttctagtgtttgtgccc
gtttgtttttctggcgaaagggttggagaggttactgggcggggtaaggctctaacgtagaggcctgcgtgcatcaagacgaatggcgtccagccagc
tgctgctttttctcaccccggagtagtcgggtgttgggggccgatgggcaggtagagcccacggctgccacgatagtgaggtagtttacaccacagta
agtcggtgttgtcctccctttactgctggacccagcatcttgaatgttccctgcatctctgggacagaccccaacctgctctttttaggacaaaggtga
gtccactcttcccagaagttgtaattgttgcaatttttgttccattgaaattaaaatataaagaataaagaattc                          2096
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TABLE 1
SEQUENCE COMPARISON BETWEEN *P. waltl* HSC90 AND HSP90 IN OTHER SPECIES

Species	Identity (%)
Homo sapiens	
HSP84β	92.1
HSP86α	82.7
Mus musculus	
HSP84β	92.1
HSP86α	82.4
G. gallus	
HSC90β	91.0
HSP90α	83.1
Drosophila melanogaster	
HSP83	75.0
Arabidopsis thaliana	
HSP81	66.1
Trypanosoma brucei	
HSP83	59.7
Plasmodium falciparum	
HSP90	56.0
Saccharomyces cerevisiae	
HSP82	59.7
HSC82	60.0
Escherichia coli	
C62,5	35.2

high level of hsc90 RNA during early oogenesis, followed by a sharp decrease in the amount of hsc90 RNA from stage II ($1.5 \cdot 10^6$ transcripts/oocyte) to stage VI ($4.3 \cdot 10^5$ transcripts/oocyte), i.e., a 1:3.5 ratio.

In situ hybridizations of the ^{35}S-labeled antisense Pw90/571 probe on ovary sections allowed us to visualize the presence of hsc90 transcripts at every oocyte stage. In young oocytes, i.e., stages I, II, and III oocytes, particularly intense labeling was observed throughout the cytoplasm, whereas no significant signal was ever detected in the nucleus. In contrast, stage VI oocytes showed weaker labeling of their cytoplasms; indeed, only a few silver grains were visible between yolk platelets, while their nuclei exhibited significant labeling. Due to the oocyte volume increase (×30) during this oogenesis period (Bonnanfant-Jaïs and Mentré, 1983), *in situ* hybridizations indicated that the hsc90 transcript concentration is much higher in young oocytes than in stage VI oocytes (Fig. 4). Indeed, taking into account both the number of transcripts for every oocyte stage (1:3.5 ratio; see above) and the volume increase of oocytes during oogenesis (×30) we could calculate that the hsc90 transcript concentration is 120-fold higher in stage I than in stage VI. Cytoplasm labeling was uniformly distributed whatever the oocyte stage. In other respects, follicular cells exhibited the same kind of labeling as oocytes (Fig. 4).

In order to determine whether the decrease in the amount of hsc90 mRNA transcripts was due to an arrest of transcription, *in situ* hybridizations of Pw90/571 [^{35}S]cRNA to the nascent transcripts of lampbrush chromosomes were performed. They resulted in significant signals on homologous loops of 4 of the 12 bivalents of the *Pleurodeles* oocyte karyotype. The labeled loop pairs were of a normal type except for bivalent VII, where labeling was detected at the level of landmark loops (Fig. 5). The same results were obtained whatever the lampbrush oocyte stage considered (stages IV to VI). All of these results thus provided evidence for hsc90 gene transcription in late oogenesis despite a concomitant decrease in the amount of hsc90 RNA.

Expression of HSC90-Related Protein during Oogenesis

HSC90 expression was investigated by immunoblots. The monoclonal antibody AC88 previously characterized by Riehl *et al.* (1985) and the polyclonal antibody

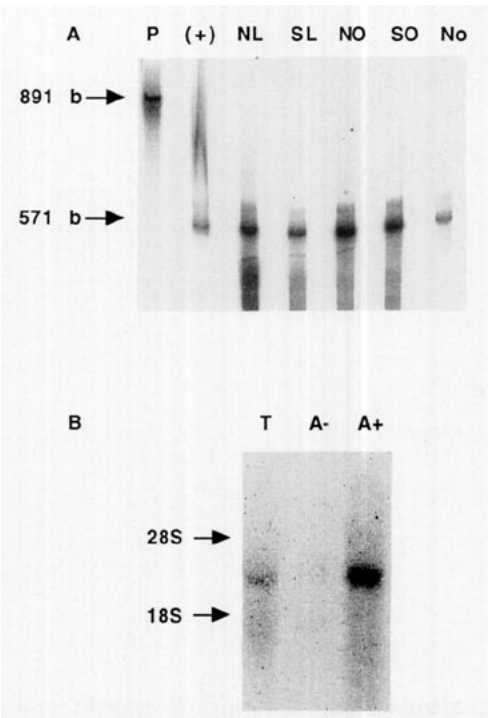


FIG. 2. (A) RNase protection assay of an 891-base ^{32}P-labeled probe consisting of Pw90/571 (571 bases) + 320 from the vector. P, probe; (+), positive control with sense Pw90/571 cRNA; NL, normal liver cells; SL, heat-shocked liver cells; NO, normal ovary; SO, heat-shocked ovary; No, normal defolliculated stage VI oocytes. Both NL and SL RNA protect the probe from degradation. Pw90/571 can therefore be assumed to be a part of a constitutive gene. No RNA protects the probe, indicating that hsc90 RNA is present in oocytes under normal conditions. (B) Northern blot hybridization of ovary constitutive RNA with [^{32}P]Pw90 cDNA probe. T, 25 μg total RNA; A$^-$, 25 μg poly(A)$^-$ RNA; A$^+$, 5 μg poly(A)$^+$ RNA; 28S and 18S indicate the migration position of rRNAs. A signal is obtained with total and poly(A)$^+$ RNA, but not with poly(A)$^-$ RNA. The hsc90 mRNA is therefore present in the ovary under normal physiological conditions.

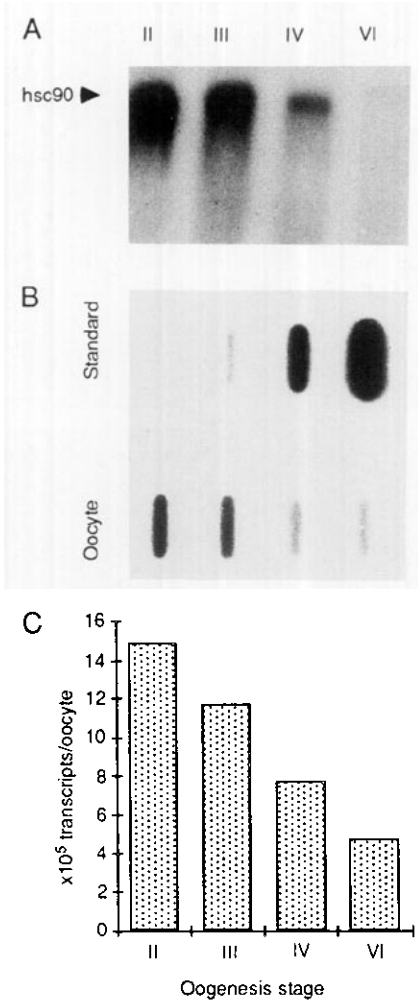


FIG. 3. (A) Northern blot hybridization of stages II, III, IV, and VI oocytes with a [^{32}P]Pw90 DNA probe. A unique hsc90 mRNA of about 3 kb was detected for each stage. (B and C) Autoradiography of an RNA slot blot of different oocyte stages hybridized with the Pw90/571 probe, and quantitation of hsp90 mRNA. Standard, synthetic sense Pw90 RNA. Left to right, 10^5, 10^6, 10^7, and 10^8 copies. Oocyte, total RNA of 4 oocytes per slot. Left to right, stages II, III, IV, and VI. By optical scanning of the autoradiogram, we determined that there are, respectively 15×10^5, 12×10^5, 8×10^5, and 5×10^5 copies of the hsc90 mRNA per oocyte at stages II, III, IV, and VI.

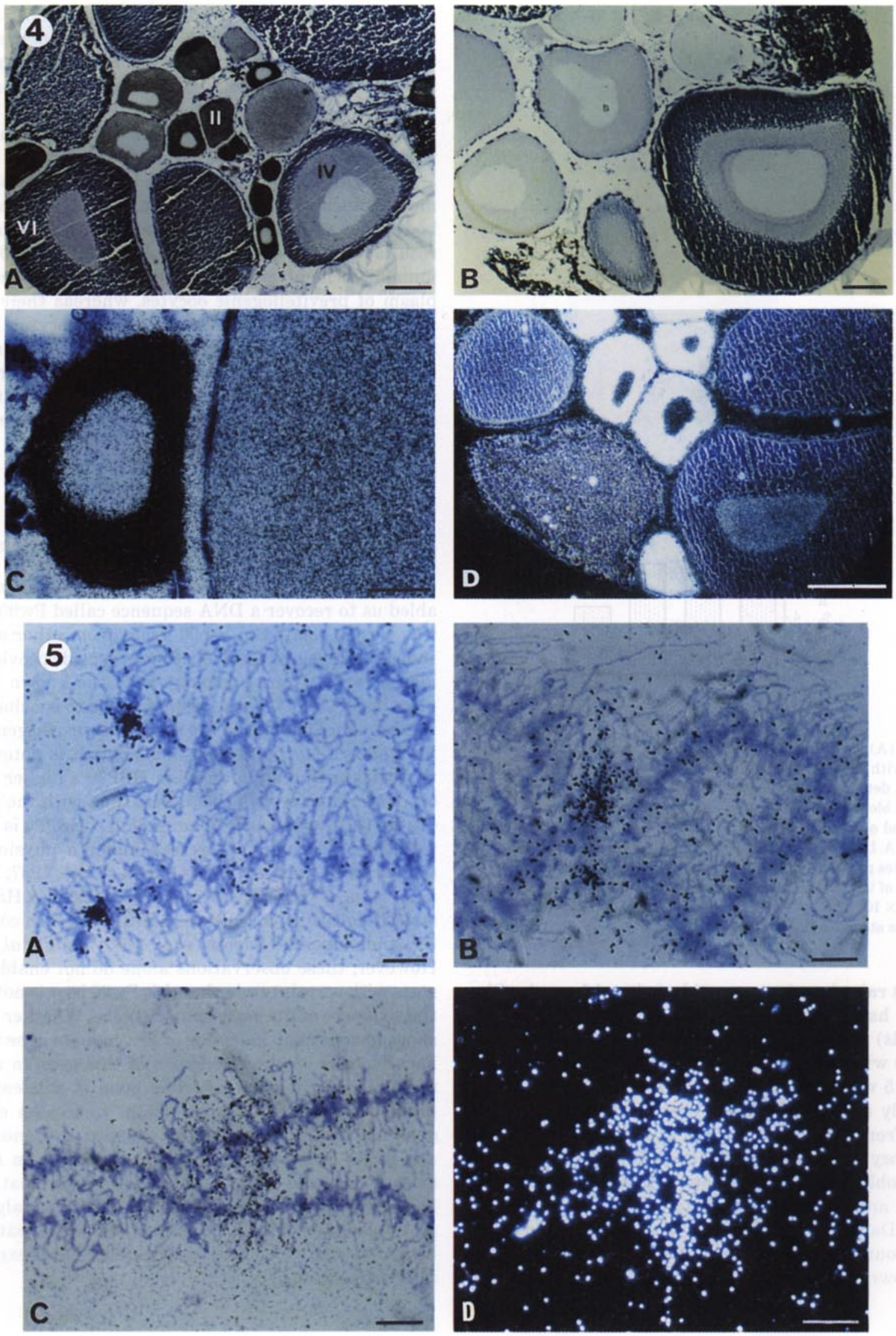
AP90Ct raised against a peptide deduced from the *Pleurodeles* hsc90 nucleotide sequence (see Materials and Methods) were used. Among oocyte proteins, one polypeptide with a molecular weight of 90×10^{-3} M_r and a p*I* of 5.5 was recognized by the two antibodies. It was probably an HSC90-related protein (Fig. 6). Total extracts from stage III and stage VI collagenase defolliculated oocytes were analyzed for their HSC90 content by immunoblotting. Immunoblots were developed with ^{125}I-labeled anti-mouse antibody to enable direct quantification. Data provided evidence for a two-fold increase in the amount of HSC90 between these two stages (data not shown). To determine whether HSC90 was a cytoplasmic and/or a nuclear protein, radioimmunoblot quantitations were carried out for these two cellular compartments of stage VI oocytes. Results provided evidence for the presence of the protein in both compartments and pointed to the fact that this protein was 10 times more abundant in cytoplasm than in nucleus (data not shown).

The localization of HSC90 during oogenesis was immunohistochemically analyzed using monoclonal antibody AC88 and polyclonal antibody AP90Ct. No positive results were ever obtained with AC88. With AP90Ct, immunohistochemical staining was significant in cytoplasm of previtellogenic oocytes, whereas their nuclei were faintly fluorescent. In vitellogenic oocytes, HSC90 appeared to be localized between yolk platelets and also in the nuclear area. Moreover, at this stage, the nucleus showed intense fluorescence (Fig. 7). Whatever the oocyte stages, follicular cells were intensely labeled, particularly in their cytoplasm (Fig. 7).

DISCUSSION

Expression of hsc90 mRNA in Pleurodeles Oocytes

Screening of a *Pleurodeles* ovary cDNA library enabled us to recover a DNA sequence called Pw90 which was closely related to hsp90 genes from other species. Indeed, analysis of the deduced polypeptide provided evidence for a strong homology with the known HSP90 proteins (Table 1). These results led us to conclude that this sequence belongs to the multigenic hsp90 gene family. Concerning the protein homology, it is noteworthy that with mammalian or avian HSP90 a higher degree of identity exists with the β form than with the α form (Table 1). Interestingly, mammalian β HSP90 is known to be more strongly expressed under a physiological temperature than α HSP90 (Barnier *et al.*, 1987; Legagneux *et al.*, 1989). Furthermore, in chicken, β HSP90 is strictly constitutive, whereas α HSP90 is both constitutive and inducible (Barnier *et al.*, 1987; Meng *et al.*, 1993). However, these observations alone do not enable us to state with certainty whether this Pw90 is or is not a partial sequence of the hsp90 or hsc90 gene. Whether a heat-shock gene is heat-inducible or is a cognate gene (hsp or hsc) depends on the regulation of this gene in normal somatic cells: a heat-inducible gene is efficiently expressed only after heat induction, whereas a cognate gene has a high basal level of expression in most cells and is not or is only weakly heat-inducible. In *Pleurodeles*, our results from test experiments for heat inducibility in somatic cells by RNase protection analysis led us to conclude that Pw90 is part of the cognate gene hsc90. We also conclude that this hsc90 gene is expressed in *Pleurodeles* oocyte.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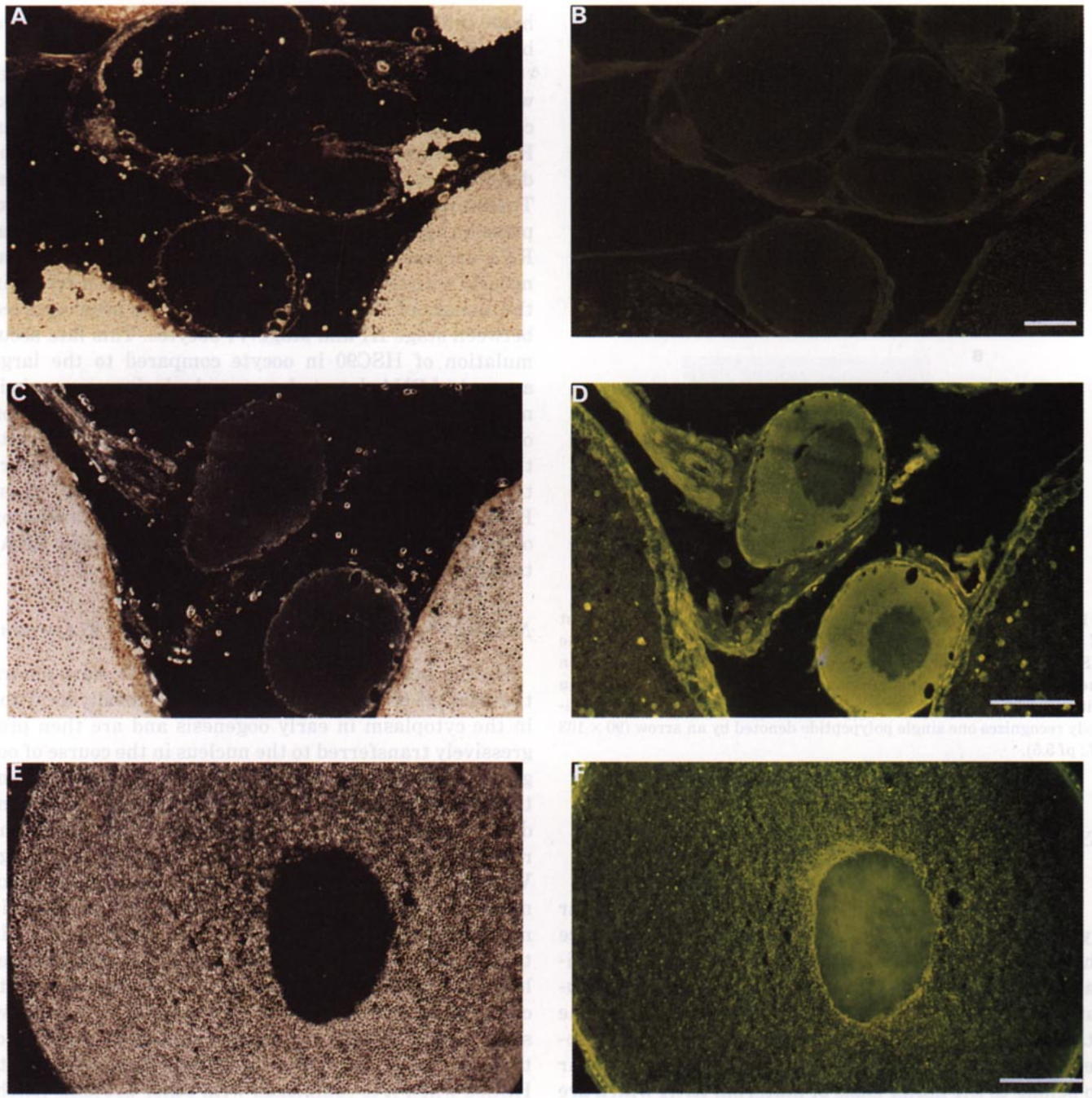

FIG. 4. *In situ* hybridization with an antisense *P. waltl* [^{35}S]Pw90/571 cRNA probe (A, C, D) or with a sense probe (B) on ovary sections. (A) and (B) represent general views hybridized with the antisense (A) or with the sense probe (B). A strong cytoplasmic signal is detected, particularly in young oocytes. The signal intensity decreases in older oocytes (stages IV and VI). No related signal is detected with the sense probe (B). (C) Detail of (A) (see asterisk) showing a stage I oocyte. Note the high concentration of silver grains in cytoplasm. (D) Detail of (A) showing stage II–III oocytes in a dark field. Note the decrease in signal intensity due to the dilution of transcripts during oocyte cytoplasm growth. Bars, 200 μm (A, B, D) or 20 μm (C).

FIG. 5. (A–D) *In situ* hybridization to the nascent transcripts on lampbrush chromosomes from stage V–VI oocytes. The probe is an antisense ^{35}S-labeled *P. waltl* Pw90/571 cRNA. Four hybridization sites are detected on lateral loops of lampbrush chromosomes in light (A, B, C) and dark (D) fields. Bars, 20 μm (A, B) or 15 μm (C, D).

FIG. 7. Immunolocalization of HSP90-related proteins on *Pleurodeles* ovary sections. (A, C, and E) Phase contrast. (B, D, and F) Corresponding immunofluorescence. (A, B) Previtellogenic oocytes (stages I and II). No significant staining is detected with preimmune serum. (C, D) Previtellogenic oocytes. Strong cytoplasmic staining is observed with immune serum. The nucleus is only faintly stained. (E, F) Postvitellogenic oocyte (stage VI). Staining is preferentially perinuclear and nuclear. Bars, 100 μm.

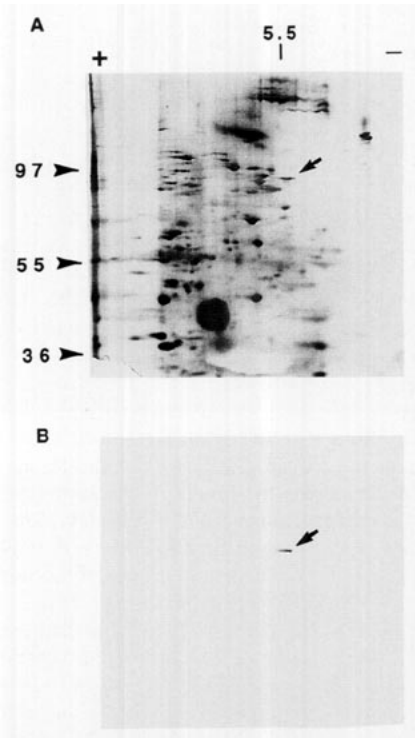

FIG. 6. (A) Two-dimensional electrophoresis of a 10000*g* supernatant of 10 stage VI oocytes of *P. waltl* (silver staining). Position of the HSC90 protein is indicated by arrows. Molecular mass designations on the left are in 10^3 M_r. (B) Corresponding immunoblot after incubation with an anti-*Achlya ambisexualis* HSP90 antibody (AC88). The antibody recognizes one single polypeptide denoted by an arrow (90 × 103 M_r; p*I* 5.5).

Expression of the hsc90 Gene in Oocytes during Oogenesis

Using Pw90/571 as a probe, we showed, by molecular hybridization, that hsc90 mRNA expression takes place throughout oogenesis. Furthermore, hsc90 RNA quantitation results provide evidence for hsc90 RNA accumulation is reached at around the lampbrush chromosome stage. Then, a decrease in the hsc90 RNA level is observed up to stage VI oocytes. Hsc90 RNAs thus appear to belong to the major class of maternal RNA which are synthesized and stored before the lampbrush stages. However, in the present case, the decrease in hsc90 mRNA transcript content in late oogenesis cannot be related to the absence of hsc90 RNA transcription, since several transcription sites were visualized on lampbrush loops of different bivalents. It can be envisaged either that hsc90 RNA transcribed on lampbrush chromosome loops may remain unmaturated and therefore not stored in oocytes; subsequently, the RNA stored very early during oogenesis and progressively degraded would not be replaced; or that this decrease may also be due to a relative ratio of synthesis to degradation of hsc90 mRNA which leads to a net decrease in the number of transcripts.

At the present time, there are no decisive arguments which support one hypothesis or the other. However, except for hsp70 RNA (Billoud *et al.*, 1993), the majority of RNA synthesized on lampbrush loops was shown to be degraded rather than matured and stored in oocytes. Therefore, during *Pleurodeles* oogenesis, hsc90 RNA expression appears to follow the classical rule of maternal RNA expression during amphibian oogenesis. Simultaneously with the decrease in hsc90 mRNA, an increase in the amount of HSC90-related protein has been reported between stage III and stage VI oocytes. This late accumulation of HSC90 in oocyte compared to the large amount of RNA detected very early during oogenesis is not surprising since, in growing oocytes, a large fraction of the synthesized mRNA is not used for immediate translation, but is dormant and/or masked for future translation (Richter *et al.*, 1984). Therefore, these HSC90 proteins do not appear to result from translation of newly synthesized RNAs, particularly from RNAs transcribed on lampbrush chromosomes.

Nuclear Transfer of HSC90 Protein in Stage VI Oocytes

On the basis of cytological analysis, we have shown that HSC90-related proteins are preferentially localized in the cytoplasm in early oogenesis and are then progressively transferred to the nucleus in the course of oogenesis. Precise biochemical studies enabled us to quantify this process. HSC90 protein is 10 times more abundant in cytoplasm than in nucleus, but due to the respective volumes of these two compartments in stage VI oocytes (volume of cytoplasm without yolk:volume of nucleus = 10:1), the HSC90 concentration is the same in nucleus and cytoplasm. We can thus conclude that, in the course of oogenesis, the HSC90 nuclear transfer leads to a concentration equilibrium between the oocyte compartments. As concerns the absence of a positive signal with AC88 by immunolocalization on oocyte sections, it has been shown that Ac88 does not bind to HSC90 when it is complexed with other proteins (Riehl *et al.*, 1985). It is thus assumed that the epitope recognized by this monoclonal antibody is masked in oocytes when proteins are not denatured.

Proteins that migrate between cytoplasm and nucleus may be involved in numerous control processes implicated in cell maintenance, growth, replication, and differentiation. In oocytes, such processes can be controlled either directly by regulatory proteins such as PCNA or c-myc (Taylor *et al.*, 1986; Gusse *et al.*, 1989; Leibovici *et al.*, 1990) or indirectly. In the latter case, molecular chaperones are specifically involved. They interact with regulatory proteins and allow their

transport in the nucleus. For example, in somatic cells, HSP70 is known to be required for nuclear transport of karyophilic proteins (Iamamoto *et al.*, 1992; Shi and Thomas, 1992; Okuno *et al.*, 1993). Recently, such a role has been suggested in amphibian oocytes in which HSP70 has been shown to mediate the nuclear transport of proteins involved in transcriptional processes (Moreau *et al.*, 1994). The behavior of HSC90 in the course of oogenesis is consistent with indirect control of transcriptional processes. Its weak nuclear concentration in early oogenesis compared to that observed in stage VI oocyte suggests a possible involvement of HSC90 in control of gene expression only in late oogenesis.

The sequence presented here appears in GenBank under Accession No. L32987.

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